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ORIGINAL PAPER

Shoot multiplication and plant regeneration in *Caragana fruticosa* (Pall.) Besser

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Abstract: Different nutrient media can affect *in vitro* culturing protocols, and experimentation under varied growth conditions is valuable in plants where *in vitro* methods are in preliminary stages. We carried out the first *in vitro* propagation studies for the endangered species *Caragana fruticosa* (Fabaceae). We evaluated various nutrient media for their impact on shoot elongation and axillary bud proliferation using different concentrations of 6-benzylaminopurine (BA) and α-naphthaleneacetic acid (NAA). Shoot elongation was evaluated based on adventitious shoot primary culture and subculture regeneration from *Caragana* seedlings. Our goal was to improve both micropropagation and regeneration in *C. fruticosa*. MS nutrient media was superior to 1/2MS macronutrients, DKW, QL, and WPM for shoot elongation and axillary shoot proliferation. Shoots grown on 1/2MS and WPM exhibited some chlorosis, and shoots on QL produced larger leavers than plants growing on normal medium. The shoot proliferation coefficient on MS media supplemented with 2.22 μM BA and 0.44 μM BA + 2.69 μM NAA was significantly higher than that with other treatments in the primary culture. Shoots on 2.22 μM BA showed a higher proliferation coefficient (3.17) than others in the subculture. Shoots were rooted on 1/2MS medium with the addition of different concentrations of NAA. The optimal concentration for rooting was 0.27 μM NAA (74%). Roots exhibited many stout and long root hairs. Survivl of established plantlets was 82% at 30 days after transfer to soil. Plants established in the green house showed normal growth and displayed no apparent morphological differences compared to stock plants.

Keywords: Endangered species**;** *Caragana fruticosa*; micropropagation; auxin; cytokinin; Basal medium

Abbreviations:

1/2MS	Half strength of MS macronutrients
BA	6-Benzylaminopurine
DKW	Driver-Kuniyuki walnut medium
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
PGRs	Plant growth regulators
QL	Quoirin and Lepoivr's medium
WPM	Woody Plant Medium

Introduction

Caragana fruticosa (Pall.) Besser is a multi-branched shrub, which is traditionally propagated with seed. *C. fruticosa* is an endangered (Qin et al. 1993) endemic species in Heilongjiang Province (Liu 1955). *Caragana* species are cold- and drought-resistant and are well adapted to high temperature and strong solar radiation (Sanczir 2003; Ma et al. 2008). *Caragana* forms nodules and fixes nitrogen with distinctive rhizobial populations (Lu et al. 2009). Toit (2009) demonstrated that *Caragana* provides nutritious and accessible forage resources for wild animals. The flowers are good sources of pollen for honey production, and the entire plant is harvested for Chinese herbal medicine (Huo et al. 2007; Cheng 2008; Xiang 2005).

Caragana is used widely for vegetation restoration and ecological construction in China (Yong et al. 2003; Jia 2001; Ma 2007; Yan 2007). Mass production of *Caragana* seedlings has been unsuccessful when using conventional methods. The procedure is slow and seed viability decreases during seed storage (Wei et al. 2000). Vegetative propagation fails because rooting is slow (Ji et al. 2006). In the last decade, *in vitro* culture researchers made progress culturing some species of *Caragana*. Organogenesis and plant regeneration were achieved using these approaches: 1) culturing axillary buds, cotyledons or hypocotyls from sterile seedlings (Zhang et al. 2005; Song et al. 2007; Hu et

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al. 2009; Huo et al. 2009); 2) culturing axillary buds from seedlings grown in a greenhouse (Niu et al. 2004; Yang et al. 2007); 3) directly inducing adventitious buds through the epicotyl or hypocotyl from mature seed (Gao and Li 1990); 4) culturing immature embryos (Guo et al. 2007); 5) sprouting young leaves from hydroponically grown stems (Zhang and Chen 1990). Plant regeneration from mature *Caragana* stems has not been reported. We used mature stems as explants in our previous research, but the pollution rate was high after sterilization and few stems sprouted.

Development of a reliable *in vitro* protocol would be of great value for the conservation and sustainable utilization of *Caragana*. However, *in vitro* propagation protocols for *C. fruticosa* have not been reported. In the present study, we evaluated the effects of growth medium composition and plant growth regulators (PGEs) on *in vitro* shoot proliferation of *C. fruticosa* to achieve an efficient procedure for microprogation.

Material and methods

Plant material, *in vitro* germination and culture conditions

C. fruticosa seeds were harvested from a wild population on Maoershan Mountain (805 m a.s.l.) in mid August 2009. Seeds were air-dried and refrigerated at 4°C until experiments were initiated. All seeds were used within 3 months of harvest.

In vitro seed germination was performed to insure sterile conditions for seedlings and seed-derived explants. Prior to incubation, seeds were sterilized as follows: (1) washed in running tap water for 3 days; (2) surface sterilized in 75% (*V*/*V*) ethanol for 30 s; (3) immersed in 0.1% (*W*/*V*) $HgCl_2$ solution containing two drops of Tween-20 for 15 min; and (4) rinsed with sterile distilled water 5 times under aseptic conditions. Following sterilization, seeds were placed on full-strength MS basal medium supplemented with 2% (*W*/*V*) sucrose and 0.6% (*W*/*V*) agar. The culture media were adjusted to pH 5.8 before autoclaving at 15-psi pressure (121°C) for 20 min. After inoculation, the cultures were incubated under culture room conditions maintained at (25±2)°C with a 16 h photoperiod, and light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool fluorescent tubes. Stout seedlings were chosen for subsequent experiments when shoots were approximately 4.5−5.0 cm long, typically after 5−6 weeks.

Basal mediums selection

Apical shoots from *in vitro* seedlings were excised into 1.0−1.5 cm segments and transferred to fresh media. The explants were inoculated on five media compositions: Murashige and Skoog (MS; Murashige and Skoog 1967), 1/2MS (half strength MS macronutrients), Driver Kuniyuki walnut (DKW; Driver and Kuniyuki, 1984), Woody Plant Medium (WPM; Lioyd and McCown 1981) and Quoirin and Lepoivre (QL; 1977), all supplemented with 2% (*W*/*V*) sucrose and 0.44 μM BA (6-benzylaminopurine), respectively, to select the optimum medium for shoot growth and proliferation. The cultures were maintained under the conditions described above. Growth observations (shoot length) and proliferations (new shoot number per inoculated explant) were recorded at 30-day intervals. MS was found most effective of the five media compositions tested (shown in Table 1). Therefore, MS was chosen as the basal medium for subsequent experiments.

Table 1. Effect of basal media supplemented with 0.44 μM BA on shoot growth and proliferation of *C. fruticosa* after 30 days culture

Medium composition	Shoot proliferation coefficient	Shoot elongation (cm)	Growth and development of shoots
MS	2.48±0.68 a	3.70 a	Many leaves, normal, green; stem green, stout and elongated, good;
1/2MS	1.92±0.56 ab	0 b	Few leaves, small, yellow; stem no elongation, rooted, cluster small, weak;
QL	1.56±0.57 b	0 b	Many leaves, big, light green; stem no elongation, cluster elongated, good;
WPM	1.55±0.25 b	0 b	Few leaves, small, yellow; stem brown, no elongation, cluster small, few, weak;
DKW	1.49±0.49 b	0 b	Many leaves, bottle green; stem no elongation, rooted, cluster small, good;

Mean values followed by different letters in each column indicate significant differences according to LSD ($p < 0.05$); Proliferation coefficient = total newly formed shoot number (> 0.5 cm) / inoculated explants; Elongation per shoot = sum of shoot elongation / total shoot number.

PGR(s) selection

Both type and concentration of PGR(s) must be optimized for shoot growth and proliferation. Apical explants from 6 week-old seedlings cultured *in vitro* on MS, were cultured on MS supplemented with various concentrations of BA (0.00, 0.44, 2.22, 4.44, 8.88 and 22.20 μM) in combination with NAA (α- naphthalene acetic acid) (0.00, 0.57, 2.69, 5.73, 10.74 and 26.85 μM). Thirty-six combinations were tested for the primary culture. Based on the results of primary cultivation (Fig 2), we narrowed the range of concentrations of both BA and NAA to select the optimum PGR concentration for subculture. The medium was solidified with 0.6% (*W*/*V*) agar and supplemented with 2% (*W*/*V*) sucrose. In the following four weeks of culture, we recorded numbers of newly formed shoots.

In vitro rooting

Small shoots (2−3 cm length) were excised from shoot cultures maintained on MS supplemented with 2.22 μM BA for 4 weeks and transferred onto fresh 1/2MS medium supplemented with different concentrations of NAA without cytokinin (0.00, 0.27, 0.57, 1.14 and 2.69 μM) for *in vitro* rooting. During the follow-

ing four weeks of culture we recorded the percentage of shoots showing root formation, and the number and length of formed roots.

Plantlet acclimatization

During the following eight weeks of culture on rooting medium, plants with two or more stout roots were washed thoroughly and transferred to plastic boxes (25 cm × 40 cm) containing a mixture of soil, vermiculite, and perlite at a ratio of 5:4:1. Ten plantlets were planted per box (total: five boxes). The boxes were covered with transparent polyethylene film to maintain high humidity in an acclimatization room and exposed to a 12 h photoperiod and a maximum daily temperature of 30°C. After 15 days, new leaves started to emerge and the polyethylene film was removed to reduce humidity. One month later, we recorded numbers of acclimated plants. Finally, the plantlets that grew in length and hardiness were transferred to plastic pots containing garden soil and held in the acclimatization room for four weeks before being moved to a greenhouse, where they were maintained at 17−22°C with natural light.

Statistical analysis

SPSS 17.0 and SigmaPlot 10.0 were used to analyze data and prepare charts. Data from each experiment were subjected to One-way Analysis of Variance (ANOVA). Least Significant Differences (LSDs) were used for means comparison. In both cases, $p<0.05$ was set as the level of significance. The treatments were arranged in a completely randomized design with five replicates per treatment and five or ten explants per replicate. Formulas were calculated as follows:

Proliferation coefficient = number of newly formed shoots (>0.5 cm) / inoculated explant;
Elongation per shoot = sum of shoot elongation / total shoot number;
Rooting coefficient (%) = total rooted shoot number ×100 / inoculated explants;
Root length (cm) = sum of root length / total root number;
Roots per shoot (no.) = total root number / rooted shoot number;
Survival rate (%) = plantlets survival number ×100 / total transferred plantlet number.

Results and analysis

In vitro shoot growth and proloferation on five basal media

Shoot proliferation coefficients and elongation per explant varied significantly by growth medium ($p < 0.05$). The mean number of shoots per explant grown on MS (2.48) was significantly greater than that on other media, with the exception of 1/2MS (1.92) (Table 1). The mean length of shoots grown on MS was significantly greater than that on other media (3.70 vs 0). Shoots on different media performed differently (Fig. 1). Shoots on MS showed normal size, green leaves and elongated shoots. In contrast, clustered shoots on 1/2MS, WPM, QL, and DKW were not elongated and were too short to be used as explants for further multiplication. Leaves exhibited some chlorosis on 1/2MS and WPM, but produced larger than normal leaves on QL.

Composition of growth media has shown a substantial effect on shoot growth and proliferation in other research (Bell et al. 2009) and larger shoots have proven to yield more available nodes following subculture, thus providing more propagation material (Papiya et al. 2010). In our research MS proved to be the optimal medium for *in vitro* culture of *C. fruticosa.* Consequently, in our subsequent experiments MS was chosen as the basal medium.

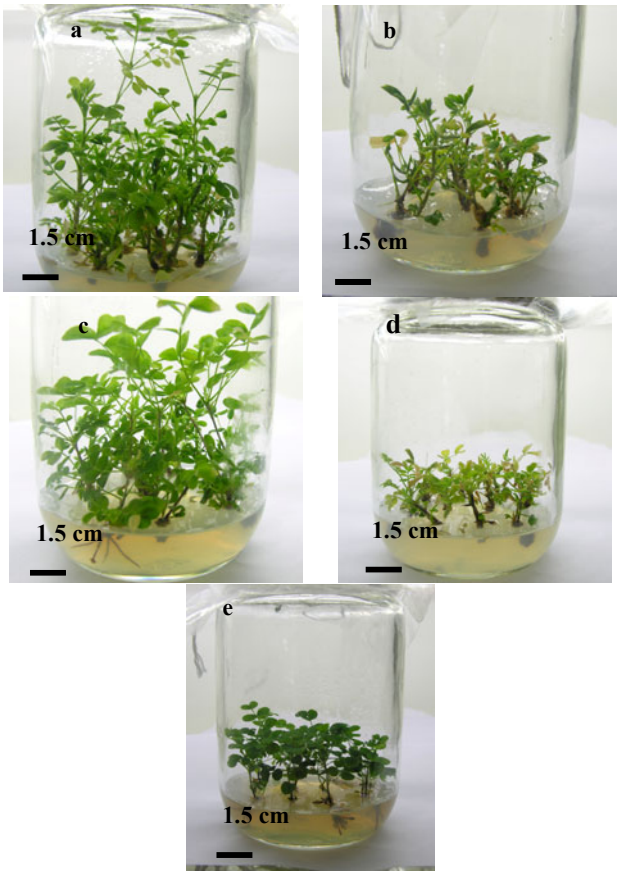

Fig. 1 Shoot development on different media supplemented with 0.44 μM BA. (a) MS, (b) 1/2MS, (c) QL, (d) WPM, (e) DKW.

Shoot proliferation in the primary culture

In vitro shoot propagation was highly influenced by PGR type and concentration. In the primary culture, the PGR-free medium was less effective for shoot growth than PGR-supplemented media. Differences in *in vitro* shoot proliferation coefficients due to PGR concentration and combinations were significant (Fig. 2). The addition of 2.22 μM BA to MS yielded the highest production of shoots per explant and MS +0.44 μM BA + 2.69 μM NAA yielded the second highest number of shoots (2.92 and 2.88, respectively). Explants initiated elongation three days after inoculation, buds began emerging seven days after inoculation,

and regenerated shoots were stout and green at the end of one month of culture (Fig. 3a). The use of BA or NAA alone can also promote growth and proliferation in *C. fruticosa*. NAA alone resulted in callused or rooted explant bases that blocked the initiation of shoots. Increased concentrations of BA, particularly those higher than 8.88 μM, resulted in yellowing of the shoot leaf, slow growth, and notable vitrification (Fig. 3b).

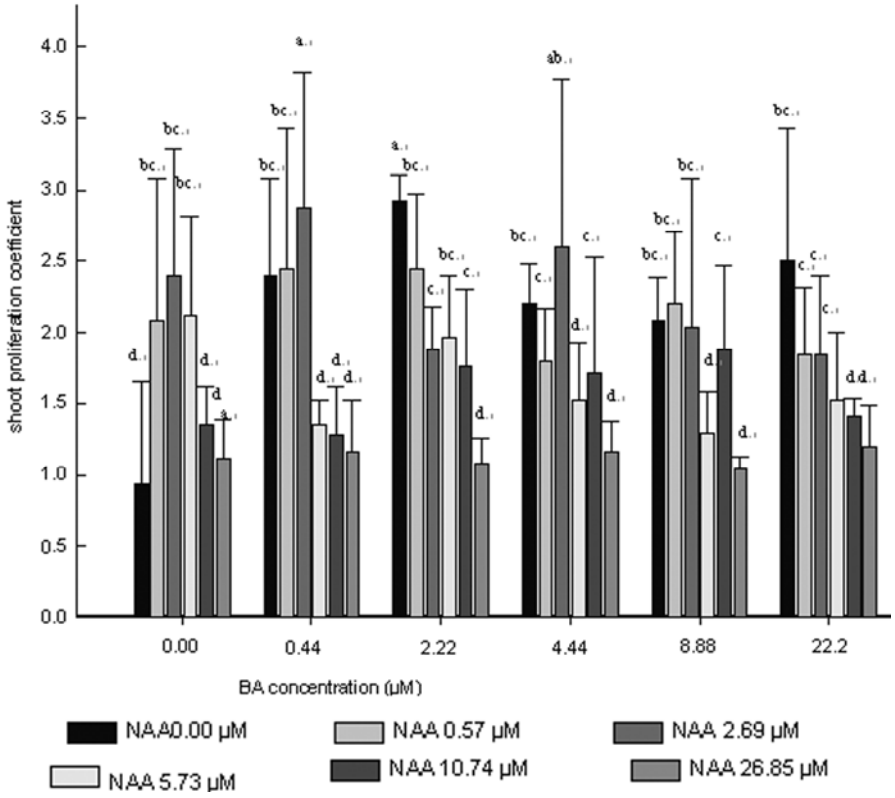

Fig. 2 Effects of different hormone combinations on *C. fruticosa* multiplication in primary culture

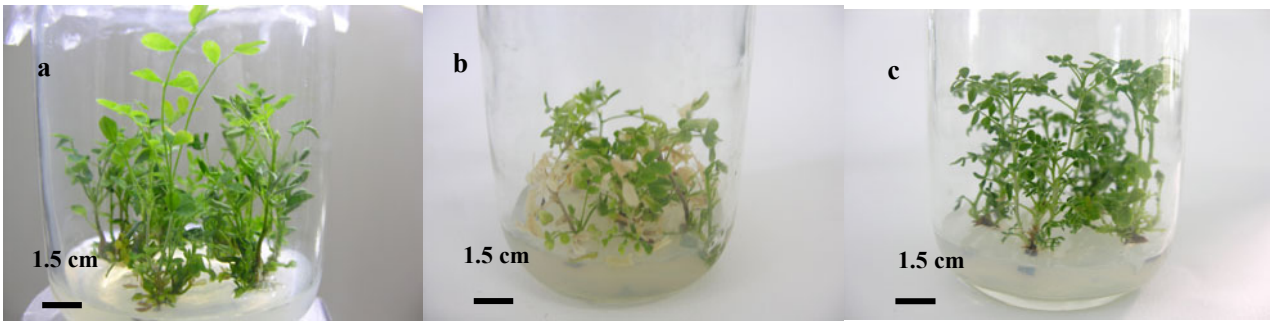

Fig 3. *C. fruticosa* shoot multiplication. (**a**) Proliferated shoots stout and bottle green in primary culture; **(b)** Yellow and vitrified shoots resulting from high concentration of BA in the primary culture ; **(c)** Healthy shoot proliferation in subculture

Shoot proliferation in subculture

PGRs were chosen based on a combination of high proliferation and limited or no calli production in the primary culture. MS medium supplemented with 2.22 μM of BA resulted in the highest proliferation coefficient (Table 2), significantly higher than all other subculture media (p<0.05) (Fig. 3c). In the subculture, the proliferation coefficient resulting from a combination of MS+ 0.44 μM BA+ 2.69 μM NAA and MS+ 4.44 μM BA + 2.69 μM NAA that performed well in two cases in the primary culture declined due to the development of calluses. Calluses inhibited shoot cluster growth and lead to plantlet death. The combination of BA and NAA did not result in a higher proliferation coefficient than the use of BA alone. BA was the primary factor for inducing *C. fruticosa* shoot proliferation, and the optimum con-

centration was 2.22 μM.

Table 2. Effects of different hormone combinations on multiplication of *C. fruticosa* in subcultures

Concentration of BA(μM)	Concentration of NAA(μM)	Shoot proliferation coefficient
0.44	0.57	1.93±1.11bc
	2.67	1.47±0.42c
	5.73	1.50±0.13c
2.22	0.57	1.38±0.41c
	2.67	1.75±0.70c
	5.73	1.77±0.33bc
4.44	0.57	1.39±0.32c
	2.67	0.27±0.31d
	5.73	1.19±0.89c
0.44	0.00	2.65±0.43ab
2.22	0.00	3.17±0.45a
4.44	0.00	2.02±0.51bc
8.8	0.00	1.62±0.27c

Mean values followed by different letters in each column indicate significant differences according to LSD ($p < 0.05$).
Proliferation coefficient = total newly formed shoot number (> 0.5 cm) / inoculated explants

The number of shoots regenerated per explant is the primary consideration for large-scale propagation (Andreu 2005). Generally, the addition of BA to the medium induces adventitious bud formation. However, high concentrations of cytokinins are reported to cause cytogenetic instability and are unsuitable for propagation, so lower BA concentrations have been applied (Peddaboina et al. 2006). In view of the above reports, the use of 2.22 μM BA in successive subcultures was avoided to reduce vitrification and the probability of somaclonal variation. The 2.22 μM BA subculture resulted in a 3.17-fold increase in shoot multiplication rate over five weeks. The multiplication coefficient increased with the use of BA and NAA, but there were no interactive effects between BA and NAA.

Rooting

Controls (that lacked NAA) showed high percentages of spontaneous rooting but roots were thin, long and slow growing. Shoots rooted in g1/2MS supplemented with NAA produced stout roots with root hairs within 30 days (Table 3). Differences in rooting rates at various NAA concentrations were highly significant ($p<0.05$). Media supplemented with 0.27 μM NAA and 2.69 μM NAA produced 70% and 74% rooting rates, respectively. However, with increasing NAA concentration more calluses formed. Roots in 0.27 μM NAA were white and stout, with many root hairs (Fig. 4). Lu (2005) reported slow formation of thin roots in *Vitis thunbergii*. Mhatre et al. (2000) determined that in *Vitis vinifera*, auxin not only induced roots but also calluses. In *Pimelea spicata*, shoots rooted *in vitro* failed to re-establish *ex vitro,* probably due to the high frequency of massive callus growth during rooting and the consequently poor vascular connection between roots and shoots (Offord and Tyler 2009). Considering the above results, 1/2MS with 0.27 μM NAA was most effective for *C. fruticosa in vitro* rooting.

Table 3. Effect of NAA concentrations in 1/2MS medium on root induction and growth of C. fruticosa after 30 days culture

NAA Concentration (μM)	Rooting rate (%)	Root length (cm)	Roots per shoot	Root status
0.00	54±20.74ab	4.56±1.16a	2.27±0.95b	white, slow, thin and long
0.27	74±18.17a	3.84±1.30ab	2.60±0.49ab	white, stout, long, many root hairs
0.57	52±8.37ab	5.10±2.24a	2.42±0.56ab	callus, yellow, stout, many root hairs, short
1.14	36±25.10b	2.95±0.90b	3.39±1.56ab	callus, brown, stout, little root hairs, short
2.69	70±18.71a	2.67±0.66b	3.65±0.77a	callus, brown and black, stout, short

Mean values followed by different letters in each column indicate significant differences according to LSD ($p< 0.05$); Rooting coefficient (%) = total rooted shoot number×100/inoculated explants; Root length (cm) = sum of root length / total root number; Roots per shoot (no.) = total root number / total shoot number.

Plant acclimatization and transfer to pots

The rooted shoots were transferred to plastic boxes after hardening (Fig. 5a). Five days later, the leaf color turned dark green and a wax coat and villi were obvious on the leaf surface; the stems were strong (Fig. 5b). At the end of 15 days, plantlets were transplanted to pots (Fig. 5c) where their survival rate was 82%. The plants were subsequently maintained in a green house and showed normal growth with no apparent morphological differences compared to stock plants (Fig. 5d). After one year growing in the green house, the regenerated plants showed 100% survival with an average height of 55.50 cm, and average root-collar diameter of 0.65 cm.

Conclusion

In vitro culture is an effective and popular method for germplasm conservation and micropropagation of endangered plants (He et al. 2007). It has the advantages of preserving healthy plant material in a small space, and it is an easy and rapid multiplication protocol for international material exchange and is cost-effective.

MS proved to be the optium medium for *in vitro* culture of *C. fruticosa*, and BA was the primary factor for inducing shoot proliferation at an optimum concentration of 2.22 μM. 1/2MS with 0.27 μM NAA was the most effective concentration for *in vitro* rooting. The survival rate of regenerated *C. fruticosa* was high in our research.

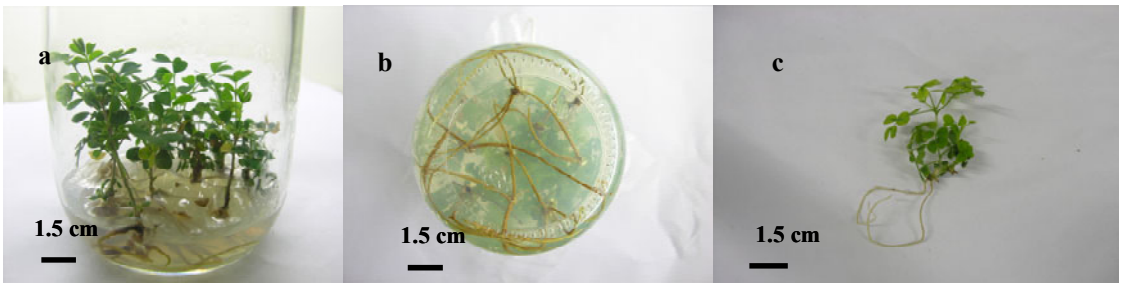

Fig. 4 *C. fruticosa* shoots rooted *in vitro*. (a) *In vitro* Rooted shoots (b) Roots in medium (c) Small plantlet before transplant

Fig. 5 Regenerated plantlets were transplanted into pots. (a) Rooted plantlets acclimated in plastic box; (b) Established plants following 15 days acclimatization; (c) Healthy growth of *in vitro* multiplicated plants at 30 days after transfer to plastic pots; (d) Regenerated plants after 6 weeks

C. fruticosa is useful in vegetation restoration and ecological construction. Our research developed an *in vitro* propagation system for *C. fruticosa* with a high shoot propagation rate. The high multiplication level was achieved through micropropagation rather than the conventional *in vivo* propagation method. After completion of our trials, the cultures were maintained in our laboratory for 14 months and were indistinguishable from stock plants in terms of morphology. This protocol can be applied to large-scale clonal propagation of *C. fruticosa*.

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